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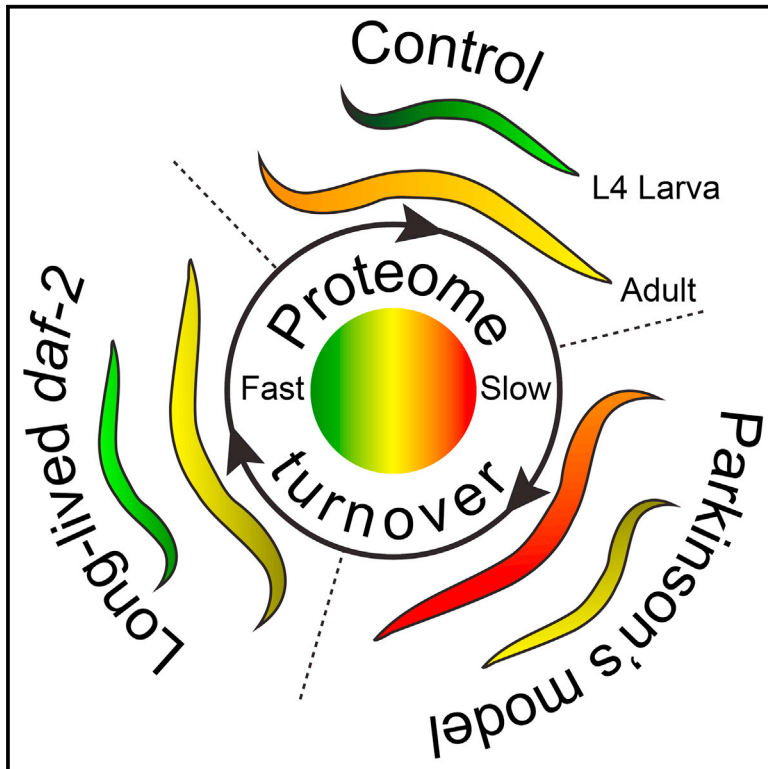
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# Cell Reports

## Proteome-wide Changes in Protein Turnover Rates in *C. elegans* Models of Longevity and Age-Related Disease

### Graphical Abstract



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### In Brief

Visscher et al. use pulsed labeling and quantitative mass spectrometry to estimate and compare proteome-wide protein turnover rates in worm models of aging. The study shows that long-lived *daf-2* worms have a faster proteome-wide protein turnover at old age, whereas a Parkinson's model has a much slower protein turnover compared to control.

### Highlights

- Proteome-wide protein turnover rates are affected in worm models of aging
- Protein turnover rate is regulated at the level of the entire proteome
- Local expression of an aggregation-prone protein affects global proteome turnover

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# Proteome-wide Changes in Protein Turnover Rates in *C. elegans* Models of Longevity and Age-Related Disease

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## SUMMARY

The balance between protein synthesis and protein breakdown is a major determinant of protein homeostasis, and loss of protein homeostasis is one of the hallmarks of aging. Here we describe pulsed SILAC-based experiments to estimate proteome-wide turnover rates of individual proteins. We applied this method to determine protein turnover rates in *Caenorhabditis elegans* models of longevity and Parkinson's disease, using both developing and adult animals. Whereas protein turnover in developing, long-lived *daf-2(e1370)* worms is about 30% slower than in controls, the opposite was observed in day 5 adult worms, in which protein turnover in the *daf-2(e1370)* mutant is twice as fast as in controls. In the Parkinson's model, protein turnover is reduced proportionally over the entire proteome, suggesting that the protein homeostasis network has a strong ability to adapt. The findings shed light on the relationship between protein turnover and healthy aging.

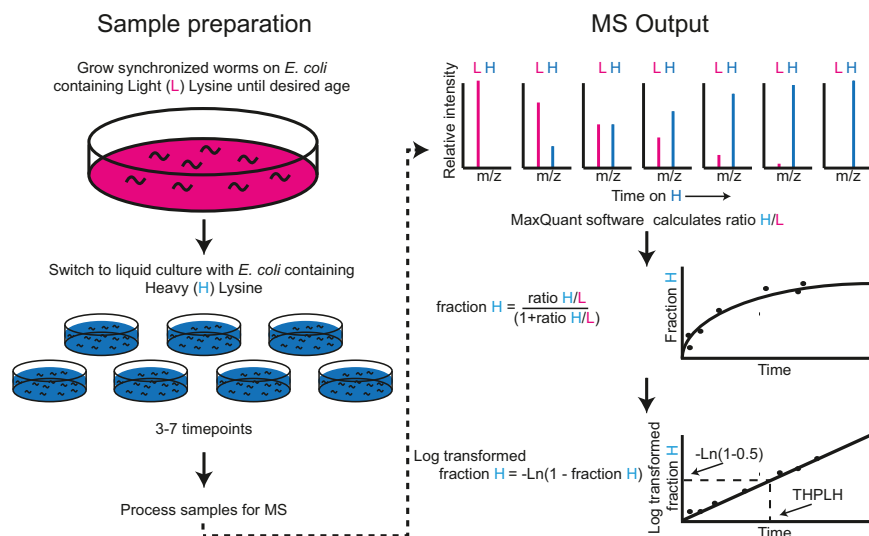
## INTRODUCTION

To ensure integrity of the soma and to adapt to different stages of life, tissues need to grow, renew, and be maintained in good condition. The balance between protein synthesis and protein breakdown (i.e., protein turnover) plays an essential role in these processes. Protein turnover is a major determinant of protein homeostasis (sometimes dubbed proteostasis) (Balch et al., 2008). The loss of protein homeostasis is one of the hallmarks of aging (Balch et al., 2008; López-Otín et al., 2013), and protein aggregation is implicated in the pathology

of age-related diseases like Parkinson's disease, Huntington's disease, cancer, and Alzheimer's disease (Powers et al., 2009). The idea that loss of protein homeostasis is implicated in aging comes mostly from studies that measure global protein synthesis and translation rates (by <sup>35</sup>S-methionine [<sup>35</sup>S-Met] incorporation and polysome profiling, respectively) during normal aging and in models of age-related disease (Depuydt et al., 2013, 2016; Hansen et al., 2007; Kirstein-Miles et al., 2013; Pan et al., 2007; Stout et al., 2013). Other studies used quantitative mass spectrometry or SDS-PAGE and western blotting to measure changes in the relative abundance of proteins in the proteome of aging model systems (Depuydt et al., 2013, 2016; Kirstein-Miles et al., 2013; Walther et al., 2015). In addition to loss of protein homeostasis during aging, it has been proposed that eventual lifespan is partly determined by the balance of allocation of resources to either maintenance of the soma or reproduction. If this were the case, we hypothesized that this would be reflected by subsets of the proteome having different rates of protein turnover.

The relatively short lifespan of *C. elegans* makes it a convenient aging model system, and many of the preceding observations regarding changes in protein synthesis with aging come from work using this model organism (Depuydt et al., 2013, 2016; Hansen et al., 2007; Kirstein-Miles et al., 2013; Pan et al., 2007; Stout et al., 2013; Walther et al., 2015). One of the best-characterized strains is the long-lived insulin receptor *daf-2* mutant, which is generally found to have decreased protein synthesis or translation activity (Depuydt et al., 2013, 2016; Hansen et al., 2007; Pan et al., 2007; Stout et al., 2013). Loss of *daf-2* is known to extend the lifespan of *C. elegans* more than 2-fold, which is, by epistasis analysis, attributed to DAF-16 function (Kenyon et al., 1993). *C. elegans* is also an established model to study age-related diseases like Parkinson's disease. For instance, overexpression of human  $\alpha$ -synuclein coupled to yellow fluorescent protein (YFP) in the body-wall muscles of *C. elegans* leads to age-dependent





**Figure 1. Workflow for the Determination of Protein Turnover in *C. elegans***

Synchronized L1 stage worms were grown on OP50 bacteria (with normal, light Lys) until stage L4 or until adult day 5, depending on the experiment. At this stage, worms were simultaneously placed on HT115 bacteria labeled with heavy isotope Lys. Worms were harvested at three (day 5 adult), four (developing insulin mutant strains and N2), or seven (developing OW40 and OW450) time points between stage L4 and young adulthood and further processed for mass spectrometry analysis. Incorporation of heavy-labeled Lys-containing peptides over time is quantified by liquid chromatography-tandem mass spectrometry. The ratio of heavy Lys over light Lys is calculated for each protein by MaxQuant software. From these ratios, the fraction heavy Lys is calculated. Interpolation of the log-transformed fraction heavy label yields the estimated turnover rate expressed as THPLH.

accumulation and aggregate formation of this protein (van Ham et al., 2008).

However, the observed changes in protein translation rates and proteome composition when comparing, e.g., long-lived *daf-2* *C. elegans* mutants with the slightly short-lived *daf-2*; *daf-16* mutant do not necessarily mean that the combined rate of synthesis and degradation (i.e., protein turnover) is changed. The level of a protein remains de facto unaffected when a slower rate of translation is compensated for by a similar reduction in protein degradation. To get better insight into the changes in proteome-wide protein turnover, we have applied a quantitative mass spectrometry-based method that allows for the estimation of the turnover rate of each protein in the proteome and is suitable for use in *C. elegans*. This method uses a pulsed form of SILAC (stable isotope labeling by amino acids in cell culture) that allows for the quantification of the incorporation rate of stable-isotope-labeled amino acids in each protein in the proteome. This approach has been used successfully in complex organisms (Doherty et al., 2005, 2009; Pratt et al., 2002; Schwahnäusser et al., 2009). To apply this method in *C. elegans*, we took advantage of an adaptation of the SILAC protocol for *C. elegans* that feeds the worms with bacteria grown on heavy isotope-labeled ( $^{15}\text{N}_2$ - $^{13}\text{C}_6$ )-Lysine (Lys) (Larance et al., 2011).

We measured protein turnover in wild-type, *daf-2(e1370)*, *daf-16(mu86)*, and *daf-2(e1370);daf-16(mu86)* worms, as well as in a *C. elegans* model for Parkinson's disease. This allowed us to determine whether specific changes in proteome-wide protein turnover are apparent in these model strains. We measured protein turnover in worms developing from the fourth and last larval stage (L4) to young adults, as well as in day 5 adult worms. We found that protein turnover is dramatically changed in some of these strains and that changes become more overt with age. Surprisingly, in most strains tested, the observed changes in protein turnover were largely proportional over the entire proteome, suggesting that the protein homeostasis network has a strong ability to adapt and that protein homeostasis can be achieved at various global protein turnover rates.

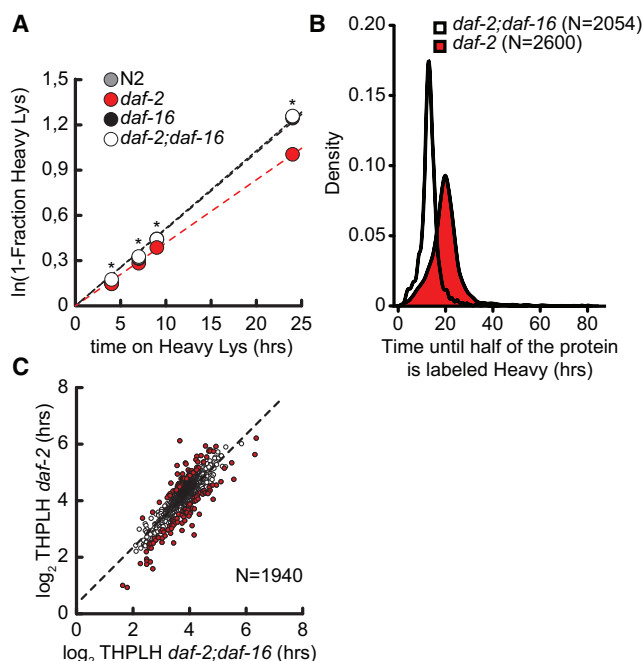
## RESULTS

### Workflow for Measuring Protein Turnover in *C. elegans*

To measure whole proteome protein turnover in worms, a method based on stable isotope labeling was set up. A summary of the experimental method is shown in Figure 1. In short, worms were synchronized and, from the desired stage (developing L4 or day 5 adult) on, fed with heavy-labeled bacteria to incorporate this label into the proteome of the worm. Animals were harvested at different time points after switching to heavy label. The heavy-to-light ratio of each protein at every time point was determined by quantitative mass spectrometry, after which these ratios were used to calculate the time it takes until half of each protein is labeled heavy (THPLH).

As an example, the fraction heavy label at each time point is plotted for 2,441 individual proteins for which THPLH was calculated in the developing wild-type N2 strain (Figure S1). From Figure S1, it is clear that most proteins incorporate heavy-labeled Lys at a more or less constant rate over the duration of the experiment.

An important step when comparing different worm strains is to assure developmental synchronization, especially because some of the used strains are known to display differences in developmental growth rate. The volume of the worms more or less doubles between L4 and adulthood; hence, small variations in synchronization have a large impact on estimated THPLH. Because of this volume-doubling, net protein synthesis will be higher than net protein degradation. This is why we use THPLH rather than protein half-lives, because the latter would likely be underestimated under these conditions. THPLH can still be compared among strains provided that within the same experiment, these strains were well synchronized. In addition to visual analysis, the quantitative mass spectrometry data can be used to assess the degree of developmental synchronization. Somatic cell division and sperm production are largely completed when the worms reach the L4 stage (the start of the experiments in developing worms) (Sulston and Horvitz, 1977); hence, protein



**Figure 2. Protein Turnover in Developing Insulin and/or IGF Signaling Pathway Mutant Worms**

(A) Average fraction heavy label of all proteins measured in the long-lived *daf-2(e1370)* strain (3,565 proteins, red circles), the short-lived *daf-2(e1370); daf-16(mu86)* strain (2,966 proteins, white circles), the wild-type N2 strain (3,213 proteins, gray circles), and *daf-16(mu86)* strain (2,704 proteins, black circles). The *daf-2(e1370)* strain incorporates heavy Lys at a slower rate in its proteome compared to the other strains ( $p < 2.2 \times 10^{-16}$  for the comparison of *daf-2* to any of the other strains according to a Benjamini-Hochberg-corrected Mann-Whitney test). The curves for the N2 and *daf-16(mu86)* strains are largely obscured by the curve for the *daf-2(e1370); daf-16(mu86)* strain (\*).

(B) Density plot of THPLH in hours for proteins for which, in at least three of four time points, a ratio of heavy over light was detected and for which the slope of a linear regression line, fitted through the log-transformed fraction heavy label versus time, could be estimated with a significance of  $p < 0.05$ . These criteria were met by 2,054 proteins for the *daf-2(e1370); daf-16(mu86)* mutant (white plot) and 2,600 proteins for the *daf-2(e1370)* mutant strain (red plot). THPLH was significantly longer in the *daf-2(e1370)* mutant ( $p < 2.2 \times 10^{-16}$ , unpaired Mann-Whitney test).

(C) THPLH in hours as in (B). The log<sub>2</sub> THPLH of each protein in *daf-2(e1370); daf-16(mu86)* is plotted against the THPLH of the same protein in the *daf-2(e1370)* mutant (1,940 proteins). The entire cloud shifts toward longer THPLH in *daf-2(e1370)* ( $p < 2.2 \times 10^{-16}$ , paired Mann-Whitney test). Proteins with a significantly longer THPLH ( $p < 0.05$ , assuming normal distribution around the mean change) compared to the average change in THPLH in either the *daf-2(e1370); daf-16(mu86)* or the *daf-2(e1370)* mutant are shaded red. These proteins were further analyzed by GO-term analysis (Table 1).

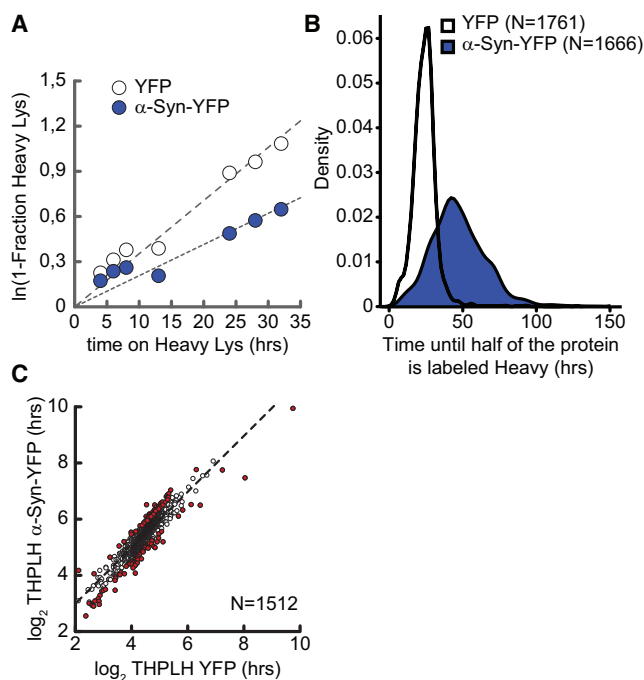
synthesis associated with cell cycle regulation is not a confounding factor. Conversely, vitellogenins are expressed only in the adult hermaphrodite intestine and are a hallmark of *C. elegans* development that can serve to determine the developmental stage of the worms (Blumenthal et al., 1984). Furthermore, gene-expression analysis has been used to cluster groups of genes that follow certain time-dependent oscillating patterns in developing *C. elegans* (Kim et al., 2013); hence, the extent of synchronization of these clusters can serve as a means to assess

the extent of developmental synchronization in our experiments. The label-free quantification intensities for vitellogenins and two oscillating gene clusters as described in Kim et al. (2013) plotted versus time were used to see which strains were synchronized properly in each experiment (Figure S2). From this assessment, we concluded that we had experiments in which strains were well synchronized to compare developing N2, *daf-16(mu86)* (CF1038), *daf-2(e1370)* (CB1370), and *daf-2(e1370); daf-16(mu86)* (DR1209) to one another and the strains expressing YFP (OW450) to those expressing  $\alpha$ -synuclein-YFP (OW40). The synchronization in day 5 adult worms was assessed in the same way and showed that these strains were synchronized (Figure S3). When making comparisons among samples, we include only those proteins for which ratios were measured in at least three time points and for which the slope of the log-transformed fraction heavy label versus time was calculated with a  $p < 0.05$  in the compared strains. From here on, this will be referred to as our comparison criteria.

### Protein Turnover in Developing Short-Lived and Long-Lived Insulin Signaling Mutant Worms

The long lifespan of the insulin signaling mutant *daf-2(e1370)* has been shown to be fully dependent on the *daf-16* gene (Kenyon et al., 1993); hence, the *daf-2(e1370); daf-16(mu86)* double mutant has a normal (or even slightly decreased) lifespan compared to wild-type worms. As mentioned, earlier studies have shown that protein translation in *daf-2(e1370)* is decreased compared to that in *daf-2(e1370); daf-16(mu86)*. We therefore compared protein turnover in these strains. When comparing *daf-2(e1370)* (3,565 proteins) with *daf-2(e1370); daf-16(mu86)* (2,966 proteins), the combined average fraction heavy label of all proteins shows that in general, the heavy label is incorporated at a slower rate in the developing *daf-2(e1370)* strain (Figure 2A). Figure 2B shows the distribution of THPLH in these strains, which was, on average, 19 hr in the *daf-2(e1370)* versus 15 hr in the *daf-2(e1370); daf-16(mu86)* mutant. In Figure 2C, THPLH of proteins measured in both strains (1,940 proteins) are plotted against each other. See Table S1 for an overview of calculated THPLH per strain and statistics for the comparisons. These figures show that THPLH between proteins varies during development from L4 to young adult but that in general, the whole proteome is shifted toward shorter THPLH in the *daf-2(e1370); daf-16(mu86)* mutant, which suggests that DAF-16 dictates global rather than individual changes in protein homeostasis. Average THPLH found for these strains and, more importantly, their relative difference are highly similar compared to those found in a replicate experiment (Figure S4, 23 hr for *daf-2(e1370)* versus 16 hr for *daf-2(e1370); daf-16(mu86)*). Protein turnover was also assessed in *daf-16(mu86)*, and as expected from the phenotype of *daf-16(mu86)* being largely identical to that of *daf-2(e1370); daf-16(mu86)*, average THPLH is similar for these strains, as well as for the wild-type N2 strain (Figure 2A; Figure S5; Table S1). Altogether, THPLH in developing *daf-2(e1370)* worms is about one-third longer compared to *daf-2(e1370); daf-16(mu86)*, *daf-16(mu86)*, and N2 worms and the changes are more or less equal over the entire proteome.





**Figure 3. Protein Turnover in Developing Parkinson's Disease Model Worms**

(A) Average fraction heavy label of all proteins measured in the Parkinson's model strain expressing  $\alpha$ -synuclein-YFP (2,239 proteins, blue circles) and in the control strain expressing YFP (2,333 proteins, white circles). Both express the transgene specifically in the body-wall muscle cells. The Parkinson's model incorporates heavy Lys at a slower rate in its proteome ( $p < 2.2 \times 10^{-16}$ , according to an unpaired Mann-Whitney test).

(B) Density plot of THPLH in hours for proteins for which in, at least three of seven time points, a ratio of heavy over light was detected and for which the slope of a linear regression line, fitted through the log-transformed fraction heavy label versus time, could be estimated with a significance of  $p < 0.05$ . These criteria were met by 1,660 proteins for the  $\alpha$ -synuclein-YFP strain (blue plot) and 1,753 proteins for the YFP control strain (white plot). THPLH was significantly longer in the  $\alpha$ -synuclein-YFP strain compared to the YFP strain ( $p < 2.2 \times 10^{-16}$ , unpaired Mann-Whitney test).

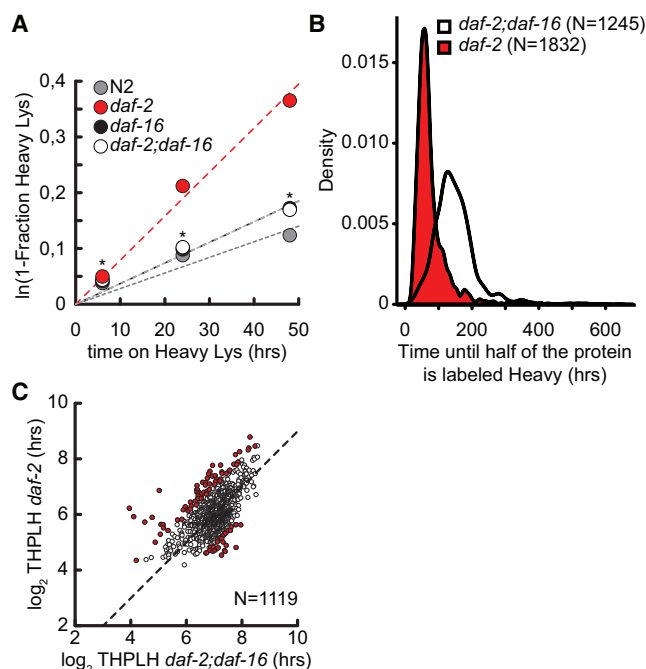
(C) THPLH in hours as in (B). The log2 THPLH of each protein in  $\alpha$ -synuclein-YFP is plotted against the THPLH of the same protein in the YFP strain (1,512 proteins). The entire cloud shifts toward longer THPLH in  $\alpha$ -synuclein-YFP ( $p < 2.2 \times 10^{-16}$ , paired Mann-Whitney test). Proteins with a significantly longer THPLH ( $p < 0.05$ , assuming normal distribution around the mean change) compared to the average change in THPLH in either the  $\alpha$ -synuclein-YFP or the YFP strain are shaded red. These proteins were further analyzed by GO-term analysis (Table 1).

To exclude that the observed large differences in measured THPLH are due to changes in clearance of light-labeled Lys in the worms (coming either from recycling of proteins or from food consumed before the switch to the heavy label), pairs of miscleaved peptides that contain two Lys instead of one were analyzed. From the ratio of the intensity of a miscleaved peptide containing two heavy Lys and the intensity of the same miscleaved peptide containing one heavy and one light Lys, the abundance of heavy and light Lys at the time of synthesis of that peptide can be calculated (Figure S6A). In developing worms, the amount of light Lys rapidly dropped over time, with

similar rates in the insulin and/or insulin growth factor (IGF) signaling mutant strains and wild-type N2. This means that potential differences in amino acid recycling are not a major factor in the observed changes in THPLH. Feeding rates were assessed to see whether the observed differences in protein turnover could be the result of or have an influence on food intake. All tested strains had similar feeding rates (Figure S7A).

### Protein Turnover in a Developing Model for Parkinson's Disease

We next investigated whether and how THPLH is altered in a model system for Parkinson's disease, again looking at animals during development from the L4 to the young adult stage. To this end, we used a strain that expresses human  $\alpha$ -synuclein-YFP in the body-wall muscle cells (OW40). These worms develop  $\alpha$ -synuclein-YFP-containing aggregates that increase with age (van Ham et al., 2008). Overexpression of  $\alpha$ -synuclein-YFP has a dramatic effect on THPLH compared to a strain only overexpressing YFP (OW450) under the same body-wall muscle-specific promoter. Henceforth, we refer to OW40 as  $\alpha$ -synuclein-YFP and to OW450 as YFP. These data come from a different experiment from that one that produced the preceding data for the insulin signaling mutants. The average fraction heavy label versus time of 2,333 proteins in the  $\alpha$ -synuclein-YFP strain and 2,239 proteins in the YFP strain are plotted in Figure 3A. The distribution of calculated THPLH represented in Figures 3B and 3C shows the comparison of 1,512 proteins that met our comparison criteria and that were identified in both the  $\alpha$ -synuclein-YFP and the YFP strains. Again, THPLH of most of the worm proteome is lengthened ( $48 \pm 31$  versus  $26 \pm 31$  hr; see Table S1 for details), even though the transgene is only expressed in the body-wall muscle, and this experiment was performed before the appearance of immobile aggregates (van Ham et al., 2008). Both strains had similar feeding rates (Figure S6A). To exclude that the observed large differences in measured THPLH are due to changes in recycling of the light label, we again assessed miscleaved peptides as described earlier (Figure S6B). Although light Lys levels drop slightly more slowly in the  $\alpha$ -synuclein-YFP strain compared to the YFP strain, these differences do not account for the large change in THPLH. For instance, after 4 hr on heavy-labeled bacteria, newly synthesized proteins contain about 88% heavy Lys in the YFP strain versus about 85% heavy Lys in the  $\alpha$ -synuclein-YFP strain. In addition, we compared the incorporation of heavy label in a developmental protein in these strains (C50F2.3) (Spencer et al., 2011). We chose C50F2.3 because it had reliable peptide counts in both strains. This protein was not yet detectably expressed at the start of the experiment but was highly expressed later in the experiment. If substantial recycling of the light Lys were to occur, we would expect to find this in the newly synthesized protein. However, incorporation of light label is very low and comparable for the  $\alpha$ -synuclein-YFP and YFP strains. The amount of remaining light Lys found in this protein after the worms were grown for 24–32 hr on heavy-labeled bacteria was comparable to what was calculated using the miscleaved peptide method. We therefore conclude that recycling is not a major confounding factor in the estimation of THPLH in the developing Parkinson's model strain (Figures S7B and S7C).



**Figure 4. Protein Turnover in Day 5 Adult Insulin/IGF Signaling Pathway Mutant Worms**

(A) Average fraction heavy label of all proteins measured in the long-lived *daf-2(e1370)* strain (3,198 proteins, red circles), the short-lived *daf-2(e1370);daf-16(mu86)* strain (2,984 proteins, white circles), the wild-type N2 strain (3,050 proteins, gray circles), and the *daf-16(mu86)* strain (3,049 proteins, black circles). In day 5 adult worms, the *daf-2(e1370)* strain incorporates heavy Lys at a faster rate in its proteome compared to the other strains ( $p < 2.2 \times 10^{-16}$  for the comparison of *daf-2* to any of the other strains according to a Benjamini-Hochberg-corrected Mann-Whitney test). The curves for the N2 and *daf-16(mu86)* strains are partially obscured by the curve for the *daf-2(e1370);daf-16(mu86)* strain (\*).

(B) Density plot of THPLH in hours for proteins for which, in all three time points, a ratio of heavy over light was detected and for which the slope of a linear regression line, fitted through the log-transformed fraction heavy label versus time, could be estimated with a significance of  $p < 0.05$ . These criteria were met by 1,245 proteins for the *daf-2(e1370);daf-16(mu86)* mutant (white plot) and 1,832 proteins for the *daf-2(e1370)* mutant (red plot). THPLH was significantly shorter in the *daf-2(e1370)* mutant ( $p < 2.2 \times 10^{-16}$ , unpaired Mann-Whitney test).

(C) THPLH in hours as in (B). The  $\log_2$  THPLH of each protein in *daf-2(e1370);daf-16(mu86)* is plotted against the THPLH of the same protein in the *daf-2(e1370)* mutant (1,119 proteins). The entire cloud shifts toward shorter THPLH in *daf-2(e1370)* ( $p < 2.2 \times 10^{-16}$ , paired Mann-Whitney test). Proteins with a significantly longer THPLH ( $p < 0.05$ , assuming normal distribution around the mean change) compared to the average change in THPLH in either the *daf-2(e1370);daf-16(mu86)* or the *daf-2(e1370)* mutant are shaded red. These proteins were further analyzed by GO-term analysis (Table 1).

### Protein Turnover Rates in Aging Worms

We next asked how proteome-wide protein turnover would change in adult worms of the preceding tested strains. We chose to investigate protein turnover at day 5 of adulthood because at this time point, egg laying has largely stopped but there is no major age-related death yet. To circumvent contamination of day 5 adult worms with eggs and progeny, they were washed with S Basal buffer over a 40  $\mu$ m pore size nylon cell strainer

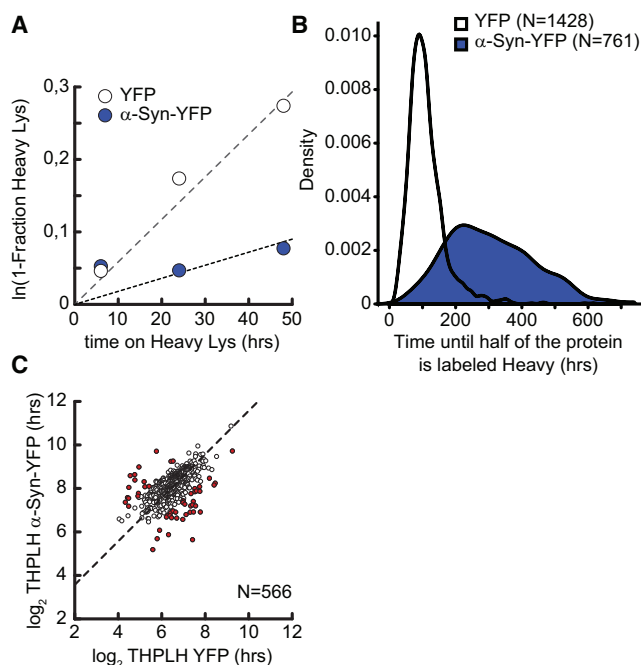
every day from day 1 to day 5 of adulthood. Visual inspection confirmed the effectiveness of this approach. For determination of THPLH by pulsed SILAC, the adult worms have the benefit that small differences in synchronization have less effect than they do in developing worms and that the total protein concentration per worm stays largely the same over the course of the experiment. However, total label incorporation is lower (Vukoti et al., 2015), which makes estimation of THPLH less reliable. Worms were pulse labeled for 6, 24, and 48 hr and harvested. As is clear from Figure S3, all strains were well synchronized over the course of the experiment.

As expected, the calculated THPLH are much longer in day 5 adult worms compared to developing worms. This might be partially explained by THPLH being affected by the high net protein synthesis in the latter due to considerable net growth in this phase of life. Nevertheless, average calculated THPLH in N2 in day 5 adult worms is considerably longer, taking  $198 \pm 102$  hr versus  $15 \pm 4.4$  hr during development. The *daf-16(mu86)* and *daf-2(e1370);daf-16(mu86)* strains have shorter but highly similar THPLH compared to N2:  $142 \pm 59$  and  $148 \pm 65$  hr, respectively. Whereas in developing worms the *daf-2(e1370)* strain had longer proteome-wide THPLH compared to N2, *daf-2(e1370);daf-16(mu86)*, and *daf-16(mu86)*, in day 5 adult worms this strain has the shortest average THPLH of  $77 \pm 53$  hr (Figure 4). When only the 316 proteins for which reliable THPLH could be calculated in all six strains are taken into account, these numbers remain largely the same (see Table S1 for an overview of calculated THPLH per strain and statistics for the comparisons). Compared to the YFP strain, with an average THPLH of  $104 \pm 57$  hr, the  $\alpha$ -synuclein-YFP-expressing strain had a dramatically increased average THPLH of  $277 \pm 155$  hr (Figure 5). Similar to what was observed in developing worms, it appeared that in all cases, the protein turnover rates of the entire proteome were affected more or less equally and there was no clear shift of a certain group of proteins. This will be discussed further.

The fraction of remaining or recycled light Lys used for protein synthesis at each time point was again calculated from the miscleaved peptides containing two Lys, as previously described. In day 5 adult worms, more light Lys was available for protein synthesis than was available in developing worms (Figure S6C). However, levels were similar when comparing *daf-2(e1370);daf-16(mu86)* and *daf-2(e1370)* mutants, suggesting that they have similar rates of amino acid recycling. In the Parkinson's model and YFP control strains, the amount of light Lys was equal after 6 hr (~50%) but then remained higher in the Parkinson's model compared to the control strain (Figure S6D). Nevertheless, if one assumes that the maximum fraction heavy label that can be achieved over a given period is the same as the fraction heavy Lys calculated from the miscleaved peptides, the  $\alpha$ -synuclein-YFP strain still has a much longer THPLH compared to the YFP control.

### THPLH Is Largely Proportionally Affected over the Proteome of Different Strains

We hypothesized that proteins associated with different biological processes might also display different turnover and that in the tested strains, average THPLH of certain biological processes could be changed more than that of others. If this were the



**Figure 5. Protein Turnover in Day 5 Adult Parkinson's Disease Model Worms**

(A) Average fraction heavy label of all proteins measured in the Parkinson's model strain expressing  $\alpha$ -synuclein-YFP (3,096 proteins, blue circles) and in the control strain expressing YFP (2,819 proteins, white circles) in the body-wall muscle cells. The Parkinson's model incorporates heavy Lys at a slower rate in its proteome ( $p < 2.2 \times 10^{-16}$ , according to an unpaired Mann-Whitney test).

(B) Density plot of THPLH in hours for proteins for which, in all three time points, a ratio of heavy over light was detected and for which the slope of a linear regression line, fitted through the log-transformed fraction heavy label versus time, could be estimated with a significance of  $p < 0.05$ . These criteria were met by 761 proteins for  $\alpha$ -synuclein-YFP and 1,428 proteins for YFP. THPLH was significantly longer in the  $\alpha$ -synuclein-YFP strain compared to the YFP strain ( $p < 2.2 \times 10^{-16}$ , unpaired Mann-Whitney test).

(C) THPLH in hours as in (B). The  $\log_2$  THPLH of each protein in  $\alpha$ -synuclein-YFP is plotted against the THPLH of the same protein in the YFP strain (566 proteins). The entire cloud shifts toward longer THPLH in  $\alpha$ -synuclein-YFP ( $p < 2.2 \times 10^{-16}$ , paired Mann-Whitney test). Proteins with a significantly longer THPLH ( $p < 0.05$ , assuming normal distribution around the mean change) compared to the average change in THPLH in either the  $\alpha$ -synuclein-YFP or the YFP strain are shaded red. These proteins were further analyzed by GO-term analysis (Table 1).

case, it could imply that in a certain strain, relatively more energy is allotted to a certain process. We reasoned that this would become apparent in the hierarchy of proteins when these are sorted from long to short THPLH. Hence, we calculated the Spearman correlation of the rank order of THPLH for the comparisons among strains, as indicated earlier. Despite the major changes in absolute THPLH observed, the Spearman's rank correlation was generally quite high in the developing insulin signaling mutant worms and was 0.933 when comparing *daf-16(mu86)* and *daf-2(e1370);daf-16(mu86)*, 0.736 when comparing *daf-2(e1370)* and *daf-16(mu86)*, and 0.751 when comparing *daf-2(e1370)* and *daf-2(e1370);daf-16(mu86)*. The Spearman's rank correlation for the developing  $\alpha$ -synuclein-YFP versus YFP strains was 0.877. These

high correlations suggest that, at least in developing worms, there is a strong hierarchy in the relative rates at which specific proteins are being turned over.

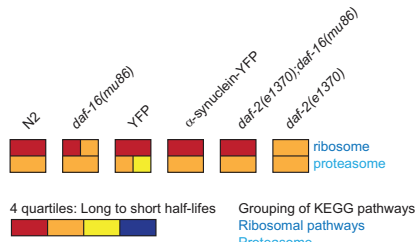
In day 5 adult worms, the same analysis showed that in general, the Spearman's rank correlations for proteins sorted by THPLH were lower when worms with different phenotypes were compared. The phenotypically similar *daf-16(mu86)* and *daf-2(e1370);daf-16(mu86)* had a Spearman's rank correlation of 0.907, and these strains both had high correlations with N2 wild-type worms (0.859 for *daf-16(mu86)* and 0.918 for *daf-2(e1370);daf-16(mu86)*). Far less correlation was observed when these strains were compared to the long-lived *daf-2(e1370)* worms: 0.380 for *daf-2(e1370)* versus *daf-16(mu86)* and 0.422 for *daf-2(e1370)* versus *daf-2(e1370);daf-16(mu86)*. Although the  $\alpha$ -synuclein-YFP worms had dramatically increased THPLH, the Spearman's rank correlations for proteome-wide THPLH, when compared to the YFP or wild-type worms, were still quite high (0.709 for  $\alpha$ -synuclein-YFP versus YFP and 0.791 for  $\alpha$ -synuclein-YFP versus N2). Because the  $\alpha$ -synuclein-YFP strain expresses the transgene only in the body-wall muscle cells, this is suggestive of non-cell autonomous regulation of relative protein turnover in this model. See Table S2 for a complete overview of correlations among strains in adult and developing worms.

Because the rank order of THPLH in *daf-2(e1370)* changed dramatically in the day 5 adult worms, we wondered whether certain biological processes were affected more than others. To test this, 316 proteins for which THPLH was determined for the day 5 adult worms in all six strains were ranked from long to short, and this list was subsequently divided into quartiles. Using ranking rather than absolute THPLH allows one to determine whether there is a different distribution of THPLH for certain biological processes in a strain and makes it possible to compare strains with large changes in overall THPLH (Figure 6).

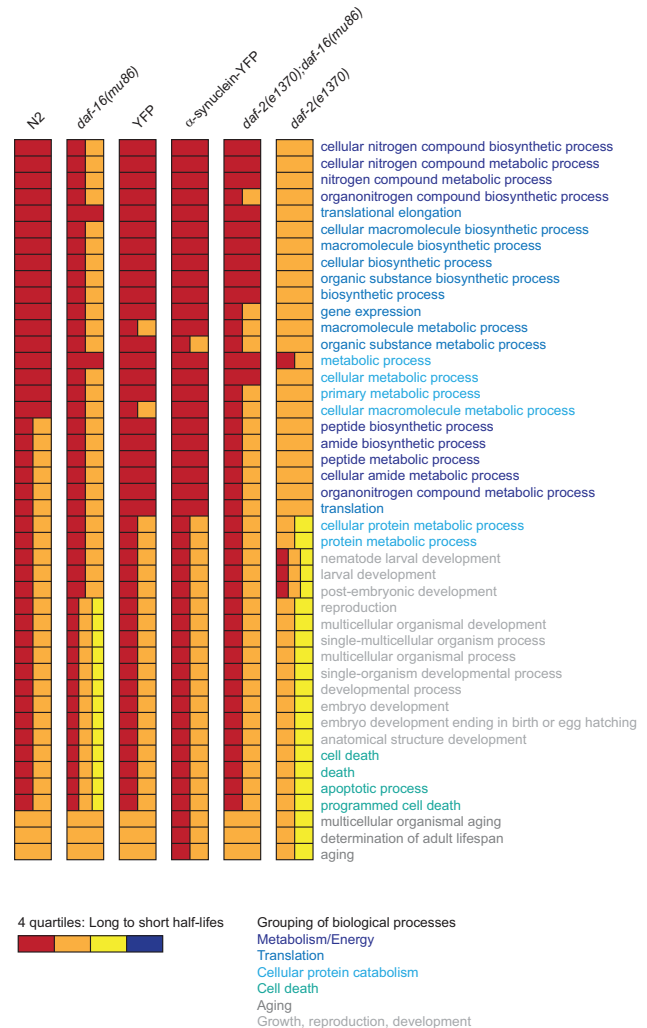
Per quartile, the overrepresented KEGG pathways (Figure 6A), biological processes (Figure 6B), and cellular components (Figure 6C) were determined through enrichment analysis. To make the comparison among strains easier, the order of the depicted terms is based on the ranking they have in the wild-type strain when sorted from long to short THPLH. Furthermore, only terms found to be significantly enriched in at least one of the four quartiles in all strains are shown. In Data S1, S2, and S3, the terms, the significance of the enrichment and the proteins, and the number of proteins included in those terms can be found. In general, the data show that proteins involved in certain biological processes are replaced at a faster rate than others. The enrichment analysis by rank order of THPLH shows that, despite large differences in average THPLH that were observed between the tested strains, the rank order of turnover rates of biological processes, KEGG pathways, or subcellular components is generally similar in most strains. However, many biological processes and components in the *daf-2(e1370)* strain are enriched in quartiles that represent shorter THPLH compared to the other strains, suggesting that in this strain, there could be a shift in allocation of resources. In both the KEGG pathways (Figure 6B) and the cellular components (Figure 6C), the terms associated with the ribosome are enriched



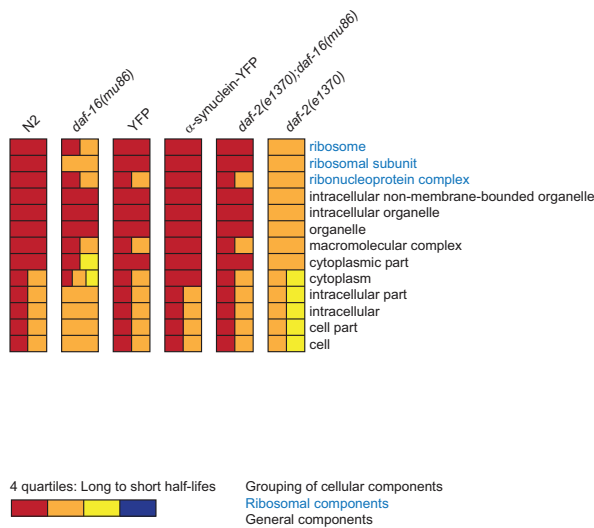
## A KEGG pathways



## B Biological process



## C Cellular component



**Figure 6. GO-Term Analysis in Day 5 Adult Worms**

(A–C) Proteins were ranked by calculated THPLH and divided into quartiles from longest (red) to shortest THPLH (blue) for each strain. Per-quartile GO-term analysis was performed to identify groups of proteins for which the ranked THPLH was enriched in a certain quartile. GO terms that are related to one another appear in the same color code. Only pathways found in all strain are depicted. GO-term analysis was performed based on (A) KEGG pathways, (B) biological processes, and (C) cellular components.

in a lower quartile in the *daf-2(e1370)* worms. This could perhaps explain why average protein turnover is faster in the day 5 adult worms in this strain. Although average THPLH of the  $\alpha$ -synuclein-YFP versus YFP is dramatically longer, the rank order of THPLH of the biological processes and components stays largely identical, again suggesting that in the  $\alpha$ -synuclein-YFP strain protein turnover is affected equally over the entire proteome.

The preceding Gene Ontology Consortium (GO)-term analysis compares 316 proteins for which THPLH was calculated in all six tested strains in day 5 adult worms. We next sought to investigate whether specific groups of proteins deviated more ( $p < 0.05$ ) in average change in THPLH when directly comparing *daf-2(e1370)* to *daf-2(e1370);daf-16(mu86)* and YFP

to  $\alpha$ -synuclein-YFP in both developing and day 5 adult worms. These proteins are shaded red in Figures 2C, 3C, 4C, and 5C. GO-term analysis was performed to identify whether pathways or processes were significantly enriched among these proteins, using all proteins in these plots as a background (see Table 1 for the results of GO-term analysis). No significant enrichment was found for the proteins with longer or shorter than average THPLH when comparing YFP to  $\alpha$ -synuclein-YFP both in developing and adult worms. In developing *daf-2(e1370)* worms, several proteins involved in the uridine 5'-diphospho (UDP)-glucuronosyl/UDP-glucosyltransferase pathway had a significantly longer THPLH compared to the average change in THPLH observed in *daf-2(e1370)* versus *daf-2(e1370);daf-16(mu86)*. This pathway has been implicated in so-called Phase II of detoxification and

**Table 1. GO-Term Analysis of Proteins with a Significantly Longer or Shorter than Average Change in THPLH, Comparing *daf-2(e1370)* to *daf-2(e1370);daf-16(mu86)* and YFP to  $\alpha$ -Synuclein-YFP**

Pathway ID	Pathway Description	Count in Network	False Discovery Rate	Gene Products	Gene Symbol
THPLH Increased in L4 <i>daf-2(e1370)</i> Compared to <i>daf-2(e1370);daf-16(mu86)</i>					
Molecular function (GO)					
GO: 0016758	transferase activity, transferring hexosyl groups	7	0.0195	B0310.5, C08F11.8.1, K08B4.3, M88.1.2, T19H12.9, Y49C4A.8a.3, ZC443.6	<i>ugt-12, ugt-16, ugt-19, ugt-22, ugt-29, ugt-46, ugt-62</i>
PFAM protein domains					
PF00201	UDP-glucuronosyl and UDP-glucosyltransferase	6	0.00138	C08F11.8.1, K08B4.3, M88.1.2, T19H12.9, Y49C4A.8a.3, ZC443.6	<i>ugt-12, ugt-16, ugt-19, ugt-22, ugt-29, ugt-62</i>
INTERPRO protein domains and features					
IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	6	0.00244	C08F11.8.1, K08B4.3, M88.1.2, T19H12.9, Y49C4A.8a.3, ZC443.6	<i>ugt-12, ugt-16, ugt-19, ugt-22, ugt-29, ugt-62</i>
THPLH Decreased in L4 <i>daf-2(e1370)</i> Compared to <i>daf-2(e1370);daf-16(mu86)</i>					
No enrichment found					
THPLH Increased in Day 5 Adult <i>daf-2(e1370)</i> Compared to <i>daf-2(e1370);daf-16(mu86)</i>					
No enrichment found					
THPLH Decreased in Day 5 Adult <i>daf-2(e1370)</i> Compared to <i>daf-2(e1370);daf-16(mu86)</i>					
Biological process (GO)					
GO: 0006869	lipid transport	5	0.00217	C04F6.1, C42D8.2a, F59D8.2, K07H8.6c, K09F5.2	<i>vit-1, vit-2, vit-4, vit-5, vit-6</i>
Molecular function (GO)					
GO: 0045735	nutrient reservoir activity	5	1.8e-05	C04F6.1, C42D8.2a, F59D8.2, K07H8.6c, K09F5.2	<i>vit-1, vit-2, vit-4, vit-5, vit-6</i>
GO: 0005319	lipid transporter activity	5	5.29e-05	C04F6.1, C42D8.2a, F59D8.2, K07H8.6c, K09F5.2	<i>vit-1, vit-2, vit-4, vit-5, vit-6</i>
Cellular component (GO)					
GO: 0043186	P granule	5	0.0241	C07H6.5.2, C18G1.4a, F56F3.1, Y18D10A.17.1, Y65B4BL.2	<i>car-1, cgh-1, deps-1, ifet-1, pgl-3</i>
GO: 0060293	germ plasm	5	0.0241	C07H6.5.2, C18G1.4a, F56F3.1, Y18D10A.17.1, Y65B4BL.2	<i>car-1, cgh-1, deps-1, ifet-1, pgl-3</i>

No significant enrichments were found when comparing developing or adult YFP and  $\alpha$ -synuclein-YFP strains.

has been shown to be transcriptionally downregulated in *daf-2(e1370)* mutants (Patel et al., 2008). No GO terms were enriched in developing *daf-2(e1370)* worms for proteins with a shorter THPLH. In the comparison of day 5 adult *daf-2(e1370)* to *daf-2(e1370);daf-16(mu86)*, several vitellogenins were enriched among proteins with a shorter than average change in THPLH. Vitellogenins have been shown to be transcriptionally downregulated in *daf-2(e1370)* worms and have been implicated in their longevity phenotype (Murphy et al., 2003). However, the protein levels of vitellogenins in *daf-2(e1370)* were also shown to be controlled by post-translational regulation (DePina et al., 2011). Another set of proteins with a reduced THPLH in day 5 adult *daf-2(e1370)* versus *daf-2(e1370);daf-16(mu86)* worms is linked to the GO terms P granule and germ plasm. These proteins are

expressed in the germline, and it has been suggested that some of these contribute to the long lifespan of *daf-2(e1370)* (Curran et al., 2009; Stout et al., 2013). P granules are involved in the post-translational regulation of RNA, which could be linked to the observed altered THPLH (Parker and Sheth, 2007).

## DISCUSSION

In past decades, several studies have suggested a mechanistic link between protein homeostasis and aging. This has been based on experiments that determined changes in protein translation rates or proteome remodeling over time in *C. elegans* models of aging and longevity (Depuydt et al., 2013, 2016; Hansen et al., 2007; Kirstein-Miles et al., 2013;

Pan et al., 2007; Stout et al., 2013). These studies showed that changes in the proteome go hand in hand with the aging process. However, these studies did not address how long each protein is present from synthesis to degradation or whether the observed proteome changes are due to an altered combined rate of synthesis and degradation of a subset of the proteome. Nevertheless, several hypotheses have been formulated in which damaged proteins need to be replaced rapidly to defer the aging process. Furthermore, it has been suggested that protein homeostasis is lost or changed during the aging process (Walther et al., 2015).

Given that changes in translation could be balanced, in principle, by changes in degradation, this would allow for rapid turnover of more abundant proteins and their continuous replacement with undamaged protein. It is therefore of added value to measure relative protein turnover rates instead of or in combination with protein abundance. In addition, accumulation followed by aggregation of proteins could be due to a change in protein turnover dynamics of only a subset of proteins. Furthermore, loss of protein turnover implies that an imbalance in the proteome develops over time, meaning that certain proteins will accumulate and that the equilibrium between certain biological processes will therefore be lost. THPLH, as measured by the pulsed stable isotope labeling and quantitative mass-spectrometry-based method presented here, gives insight into the changes in the protein turnover of each protein. This allowed us to measure how rapidly proteins involved in certain biological processes are turned over and whether and how this is changed in *C. elegans*'s model strains of longevity and age-related protein aggregation disease. This led to a number of surprising observations in this study.

First, large changes in average THPLH were observed when comparing different worm strains, and these differences were larger in day 5 adult worms than in worms developing from L4 to young adult. In developing worms, wild-type, *daf-2(e1370);daf-16(mu86)*, and *daf-16(mu86)* animals have a more or less equal protein turnover profile: on average, 50% of all proteins are heavy Lys labeled about 15 hr after the start of the pulse in these strains. The long-lived *daf-2(e1370)* worms have a slower protein turnover during development, and it takes about 19 hr to reach the same amount of heavy label after the start of the pulse. The  $\alpha$ -synuclein-YFP-expressing Parkinson's model worms need more than twice as much time before 50% of the proteome has incorporated the heavy label compared to the strain expressing YFP only, and developmental time, feeding rate, and recycling of light-labeled Lys do not explain the observed difference in this strain. Hence, during development from L4 to young adult, before any aging phenotype is apparent, the deletion or overexpression of a single gene can have dramatic effects on protein turnover of the entire proteome. We conclude that a slower protein turnover during development occurs both in a model of longevity and a model of aging and hence does not necessarily predict eventual lifespan. Our observations regarding proteome-wide protein turnover in developing insulin signaling mutant worms are similar to those recently obtained by the Braeckman group (Dhondt et al., 2016). This study had a comparable approach but used heavy nitrogen labeling and day 2 adult worms in a sterile *glp-4* background. Like us, the

authors came to the conclusion that protein turnover is slowed in the *daf-2(e1370)* versus *daf-2(e1370);daf-16(mu86)* strains. The same study confirmed that protein turnover rate measured by pulsed labeling and quantitative mass spectrometry nicely correlates with classical  $^{35}\text{S}$ -Met-based methods. Furthermore, this study used time-lapse imaging of a photoconvertible Dendra2 fusion protein to corroborate the observations on differences in THPLH in the *daf-2(e1370)* versus *daf-2(e1370);daf-16(mu86)* background.

In day 5 adult worms, average THPLH is much longer, which is in line with observations by Vukoti et al. (2015). This study used a pulsed SILAC-based approach similar to ours to study protein turnover in wild-type worms during adulthood and found that protein turnover drastically dropped after day 5. No comparison was made with strains with longevity or aging phenotypes in this study. We found that at this stage of life, the long-lived *daf-2(e1370)* worms had protein turnover rates that more closely resembled those observed in developing worms compared to the other strains. Unexpectedly, the *daf-16(mu86)* and *daf-2(e1370);daf-16(mu86)* strains, both of which have a slightly reduced lifespan, had faster protein turnover rates compared to wild-type worms, albeit still much slower than *daf-2(e1370)*. A study by the Braeckman group (Depuydt et al., 2016) describes  $^{35}\text{S}$ -Met pulse-chase experiments and showed that in *daf-2(e1370);daf-16(mu86)*, worm protein translation and protein degradation rates are initially higher than those in *daf-2(e1370)* but that they drop drastically between day 2 and day 5 of adulthood. In *daf-2(e1370)* worms, rates of both processes remain largely the same over this period. These observations could potentially explain our observations that in day 5 adult worms, *daf-2(e1370)* animals have a lower THPLH than do *daf-2(e1370);daf-16(mu86)* animals, whereas the Braeckman group (Dhondt et al., 2016) finds the opposite in day 2 adults; the latter is similar to what we find in developing L4 worms. The day 5 adult  $\alpha$ -synuclein-YFP strain had by far the slowest average THPLH, although part of this might be explained by the higher levels of remaining light label, which could, for instance, come from increased protein recycling. At first, this may seem to contradict earlier (Depuydt et al., 2013) and recent (Walther et al., 2015) studies that find a link between lifespan and protein abundance, but again, protein abundance and protein turnover rates do not necessarily correlate.

Second, our data indicate that the protein homeostasis network has a remarkable plasticity. Although large differences in protein turnover were found in the analyzed strains at day 5 of adulthood, the THPLH rank order in these strains remains largely unaffected, except for in the *daf-2(e1370)* worms. The latter could be in accordance with the idea that long-lived animals allocate energy toward maintenance of the soma rather than to production of offspring (Kirkwood, 2005). We found that ribosomal proteins had a relatively faster turnover rate in this strain, which could mean that the synthesis of new ribosomes is important for maintenance of the remainder of the proteome. Proteins involved in the biological processes of aging and determination of adult lifespan are both significantly and oppositely enriched in a quartile with shorter THPLH for *daf-2(e1370)* and longer THPLH for  $\alpha$ -synuclein-YFP (Figure 6B). Groups of proteins previously implicated in *daf-16*-dependent longevity in *daf-2* mutants (vitellogenins,

proteins involved in the UDP-glucuronosyl/UDP-glucosyltransferase pathway, and P granule proteins) were also found to have changed protein turnover rates.

Third, we conclude that protein turnover is regulated in a non-cell autonomous fashion, at the level of the whole organism. The best example presented in this study that illustrates this finding is the Parkinson's model that expresses human  $\alpha$ -synuclein-YFP exclusively in the body-wall muscles, which make up less than 10% of the worm's 959 somatic cells (Altun and Hall, 2009). THPLH in this worm is more than twice as long for all proteins in the organism in both developing and day 5 adult worms, meaning that the muscle-specific expression of the transgene affects protein homeostasis networks throughout the organism. Non-cell autonomous regulation of protein homeostasis has been described previously: Van Oosten-Hawle et al. (2013) found that disturbed protein homeostasis led to changes in chaperone expression in other tissues. In future experiments, it will be interesting to see whether expression of aggregation-prone proteins in different tissue types can also exert this whole-body response in altered protein turnover. Another mechanistic explanation for the observed global changes in protein turnover is a difference in the rates of feeding or heavy label absorption by the intestine (although we did not measure differences in food intake in the developing worms). The used *daf-2(e1370)* strain has been shown to have a lowered metabolism (Depuydt et al., 2014), but it is not clear whether this would be a rate-limiting factor for heavy label incorporation in the proteome. The described differences in metabolism would imply a slower rather than a faster protein turnover like we found here. Finally, even if the observed differences in protein turnover could be explained by lowered food intake, it does not mean that these differences are not important: food intake and absorption are likely part of the intricate protein homeostasis network.

Collectively, our data suggest that changes in proteome-wide protein turnover occur over the life of the organism but that there is no simple link between protein turnover rates and eventual adult lifespan. Measured average THPLH does not fully correlate with the eventual lifespan of the tested strains. However, in day 5 adult worms, which are at the start of post-reproductive aging, the fastest protein turnover is seen in the strain with the longest lifespan, whereas a slow protein turnover is observed in a model of age-related disease. In accordance with the observed hierarchy in THPLH, we propose that overall protein turnover must be tightly regulated to achieve protein homeostasis. One could imagine that in the case of slower protein turnover, damaged proteins are not cleared rapidly enough, leading to accumulation of dysfunctional proteins. However, when protein turnover is faster, it means that both synthesis and degradation pathways are working at high speed. This could potentially make the protein homeostasis network vulnerable, because if in this situation one of the two pathways were slightly delayed, it would cause rapid proteome imbalance. The method described in this study may be used to further elucidate the interplay between protein turnover and aging.

## EXPERIMENTAL PROCEDURES

The experimental procedures are summarized in Figure 1. See Supplemental Information for full details.

## ACCESSION NUMBERS

The accession number for the raw mass spectrometry proteomics data reported in this paper is PRIDE: PXD004561.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, two tables, and three data files and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.08.025>.

## AUTHOR CONTRIBUTIONS

M.V., S.D.H., M.H.E.W., H.R.V., and T.B.D. designed the experimental setup. M.V., M.H.E.W., S.D.H., and T.B.D. prepared *C. elegans* mass spectrometry samples. H.R.V. and R.M.v.E. performed mass spectrometry measurements and analysis. M.V., S.D.H., M.H.E.W., H.R.V., and T.B.D. performed data analysis. P.K. aided in the statistical analysis. E.A.N. and H.M. provided advice on the Parkinson's model strain. I.D. and B.P.B. provided advice and helped with validation of the data. B.M.T.B. provided tools and reagents and discussed the data. M.V., S.D.H., H.R.V., and T.B.D. wrote the paper.

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